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(54) Title: PHARMACEUTICAL COMPOSITIONS CONTAINING BACTERICIDAL PERMEABILITY INCREASING PROTEIN AND A SURFACTANT

(57) Abstract

Polypeptide pharmaceutical compositions having improved stability and resistance to aggregation, particle formation and precipitation comprising a polypeptide pharmaceutical and poloxamer surfactants alone, or in combination with polysorbate surfactants. Preferred polypeptides stabilized are bactericidal/permeability increasing (BPI) protein, biologically active fragments of BPI, biologically active analogs of BPI, and biologically active variants of BPI.

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PHARMACEUTICAL COMPOSITIONS CONTAINING BACTERICIDAL PERMEABILITY INCREASING PROTEIN AND A SURFACTANT

This is a continuation-in-part of U.S. Patent Application Serial No. 08/012,360 filed February 2, 1993.

BACKGROUND OF THE INVENTION

The present invention relates generally to pharmaceutical compositions and more specifically to improved protein and polypeptide pharmaceuticals for use as parenteral drugs. Recent advances in the development of genetic engineering technology have made a wide variety of biologically active polypeptides available in sufficiently large quantities for use as drugs. Polypeptides, however, can be subject to particulate formation and loss of biological activity by a variety of chemical and physical means including denaturation due to heating or freezing and by exposure to extreme pH or other chemical degradation.

Particulate formation and loss of biological activity can also occur as a result of physical agitation and interactions of polypeptide molecules in solution and at the liquid-air interfaces within storage vials. It is believed that the polypeptide molecules adsorb to an air-liquid interface, unfolding to present hydrophobic groups to air with the hydrophilic groups immersed in the aqueous phase. Once so positioned at the surface, the polypeptide molecules are susceptible to aggregation, particle formation and precipitation. It is also believed that further conformational changes can occur in polypeptides adsorbed to air-liquid and solid-liquid interfaces during compression-extension of the interfaces such as occurs from agitation during transportation or otherwise. Such agitation can cause the protein to entangle, aggregate, form particles and ultimately precipitate with other adsorbed proteins.

Particle formation due to surface denaturation can be somewhat controlled by appropriate selection of the dimensions of storage vials and by

minimizing the air volume (headspace) in those vials. In this regard, partially filled containers represent the worst case for vibration induced precipitation.

Particle formation can also be controlled by incorporation of surfactants into the protein containing composition in order to lower the surface tension at the solution-air interface. Classic stabilization of pharmaceuticals by surfactants or emulsifiers has focused on the amphipathic nature of molecular groups containing both hydrophilic and hydrophobic properties within the surfactant molecule. Thus, the art teaches that one can make a stable solution of immiscible molecules such as oil-in-water or water-in-oil by selecting an appropriate surfactant as a compatibilizer. One example is the stable emulsification of soybean oil using poloxamer 188 (PLURONIC F-68, BASF Wyandotte Corp., Parsippany, NJ). Another example is the use of polysorbate 80 (TWEEN 80, ICI Americas, Inc., Wilmington, DE) to emulsify oil-soluble vitamins A, E and K in aqueous solution for administration via oral and vascular routes. Work by Krantz, et al., "Sugar Alcohols - XXVIII. Toxicologic, Pharmacodynamic and Clinical Observations on TWEEN 80," Bull. of the School of Med., U. of MD., 36, 48 (1951) laid the groundwork leading to the listing of polysorbate 80 as a drug ingredient for which USP/NF requirements have been established in U.S. Pharmacopeia XXII.

Of interest to the present invention is the work related to use of polysorbate 80 for stabilization of antibody-based product formulations as described in Levine, et al., J. Parenteral Sci. Technol., 45, 3, 160-165 (1991). This work disclosed that the amount of surfactant required for stabilization was in excess of the theoretical minimum required to reduce surface tension. The work further showed that the need for excess surfactant beyond the theoretical minimum could be attributed to (1) the concentration required to maintain an intact protective layer on a turbulent interface during random shaking; and (2) to surfactant loosely associated with protein and bound to container walls.

Regulatory requirements limit the types and specific identities of surfactants that can be incorporated into parenteral compositions for injection into the human body. Generally accepted surfactants having a history of use and listed in the U.S. Pharmacopoeia XXII include poloxamer and polysorbate polymers. However, either of these alone may provide less than complete stabilization for the pharmaceutical compositions when used at concentrations of 0.1% or lower. Elevated concentrations of surfactant may pose increased risk of toxic effects, earlier onset of hemolysis, and observed changes in neutrophils and platelets, both of which are involved in blood complement activation. The highest safe concentration for poloxamer 188 in approved parenteral solutions is 2.7% when it is used in limited doses as a blood substitute and is diluted as much as 10 fold in the bloodstream. Similarly, polysorbate 80, approved in parenteral solutions for over 20 years, is rarely used in concentrations greater than 0.1% in solution volumes of 100 mL or more. Krantz et al., supra, identifies the onset of hemolysis in the dog for a polysorbate concentration of 0.1% at 90 minutes. Neonatal deaths have been associated with the use of polysorbate 80 at concentrations of greater than 1%. Accordingly, there exists a need in the art for pharmaceutical compositions providing improved protein stability which comprise only those components which are regarded as safe and are included in parenterals approved by regulatory authorities for commercial use.

SUMMARY OF THE INVENTION

The present invention relates to pharmaceutical compositions of polypeptides and is directed to the discovery that poloxamer surfactants and combinations of poloxamer surfactants with polysorbate surfactants enhance the solubility/stability of bactericidal/permeability increasing (BPI) protein, biologically active fragments of BPI, biologically active analogs of BPI, and biologically active variants of BPI (produced by either recombinant or nonrecombinant means) in aqueous solution. The invention particularly provides

for solubilization/stabilization of bactericidal/permeability increasing proteins which are biologically active amino-terminal fragments of BPI or analogs and variants thereof. Amino-terminal fragments of BPI, such as those designated $rBPI_{23}$ or any amino-terminal fragment comprising from about the first 193 to about the first 199 amino-terminal amino acid residues of BPI, are believed to be particularly susceptible to loss of stability in aqueous solution.

The present invention is directed in particular to the discovery that a combination of two specific types of surfactants provides a surprising improvement in protein stability to pharmaceutical compositions compared to either surfactant alone. Specifically, it has been found that a pharmaceutical composition comprising the combination of a poloxamer (polyoxypropylene-polyoxyethylene block copolymer) surfactant and polysorbate (polyoxyethylene sorbitan fatty acid ester) surfactant provides improved stability and resistance to aggregation, particle formation and precipitation of protein pharmaceutical agents. The combination of these two types of surfactants provides improved stability and resistance to surface denaturation, aggregation, particle formation and precipitation compared with either surfactant alone.

The poloxamer surfactant component is preferably present in a concentration of from about 0.01% to about 1% by weight with a concentration of 0.1% to 0.2% by weight being preferred to stabilize protein solutions comprising less than or equal to 2 mg/mL. The polysorbate surfactant component is preferably present in a concentration of from about 0.0005% to about 1% by weight with a concentration of 0.002% by weight being preferred. Most preferred is the combination comprising 0.1% to 0.2% by weight of poloxamer 188 and 0.002% by weight polysorbate 80. This combination is particularly useful for preventing particle formation of extremely degradation sensitive proteins such as bactericidal/permeability increasing protein (BPI) but is also useful for promoting the stability of other polypeptide pharmaceuticals. It is contemplated that the combination of poloxamer and polysorbate surfactants may

be used alone or in combination with additional surfactants. Moreover, the invention is not limited to a single poloxamer surfactant in combination with a single polysorbate surfactant and can include one or more poloxamer surfactants in combination with one or more polysorbate surfactants.

A further aspect of the invention relates to the discovery that a poloxamer surfactant is particularly useful for the solubilization/stabilization of compositions comprising an aqueous solution of BPI protein or biologically active fragments, analogs, or variants of BPI protein (produced by recombinant or nonrecombinant means). The invention provides a method of solubilizing/ stabilizing such polypeptides by contacting the polypeptide with a poloxamer surfactant. Without being bound by a theory of the invention, it is believed that poloxamer surfactants stabilize BPI protein products not by a mechanism involving lowering the surface tension of the aqueous solution, but, at elevated temperatures, by stabilizing unfolded and partially unfolded BPI protein molecules and preventing precipitation of those molecules.

Preferred poloxamer surfactants are characterized by a HLB value greater than about 14 and a surface tension between 10 and 70 mN/m as measured in aqueous solution at room temperature and at a concentration of 0.1%. More preferred is a poloxamer surfactant which has an HLB value between about 25 and 35 and has a surface tension between 30 and 52 mN/m as measured in aqueous solution at room temperature and at a concentration of 0.1%. Most preferred is poloxamer 188 available commercially as PLURONIC F-68 (BASF Wyandotte, Parsippany, N.J.) which is characterized by a surface tension of 50 mN/m and by an HLB value of 29.

A preferred polysorbate surfactant preferably has a surface tension between 10 and 70 mN/m as measured in aqueous solution at room temperature and at a concentration of 0.1%. More preferably, the polysorbate surfactant is characterized by a hydrophilic/lipophilic balance (HLB) value of about 15 and by a surface tension between 40 and 50 mN/m as measured in aqueous solution at

room temperature and at a concentration of 0.1%. Most preferred is polysorbate 80 (sorbitan mono-9-octadeconoate) which is available commercially as TWEEN 80 (ICI Americas Inc., Wilmington, Del.).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph depicting survival results over time of an actinomycin-D sensitized mouse model;

Fig. 2 is a graph depicting survival results according to BPI dose in an actinomycin-D mouse model; and

Fig. 3 is a graph depicting turbidity measurements of various BPI proteins with and without the preferred surfactants of the invention.

Fig. 4 is a graph depicting surface tension measurements of $rBPI_{21}\Delta cys$ solutions with varying surfactant concentrations of polysorbate 80 (PS80) and poloxamer 188 (F68).

Fig. 5 is a series of graphs of differential scanning calorimetry results of $rBPI_{21}\Delta cys$ with various concentrations of the surfactant poloxamer 188 (F68).

Fig. 6 is another series of graphs of differential scanning calorimetry results of $rBPI_{21}\Delta cys$ with various concentrations of poloxamer 188 (F68)

Fig. 7 is a plot of the denaturation and precipitation temperatures of $rBPI_{21}\Delta cys$ over varying concentrations of the surfactant poloxamer 188 (F68).

Fig. 8 is a series of graphs of differential scanning calorimetry results of $rBPI_{21}\Delta cys$ with various concentrations of polysorbate 80 (PS80) alone or in combination with 0.1% poloxamer 188 (F68) by weight.

Fig. 9 is a set of graphs of differential scanning calorimetry results of $rBPI_{21}\Delta cys$ with the surfactant polysorbate 80 (PS80) at two different concentrations.

Fig. 10 is a set of graphs of differential scanning calorimetry results after a solution of $rBPI_{21}\Delta cys$ and poloxamer 188 (F68) was heated to a temperature higher than the denaturation/unfolding temperature but lower than the precipitation temperature, and then was cooled down for repeat scanning.

DETAILED DESCRIPTION

The present invention provides improved methods and materials for maintaining the stability of polypeptide pharmaceuticals and preventing surface denaturation of such biologically active polypeptides. Specifically, the invention relates to the discovery that a combination of two specific types of surfactant molecules provides synergistic improvements in stabilization from surface denaturation of polypeptide pharmaceuticals. The invention also relates to the discovery that poloxamer surfactants have unique properties in the solubilization/stabilization of BPI-related proteins. While specific embodiments of the invention are directed to stabilization of bactericidal/permeability increasing protein (BPI) and biologically active fragments and/or analogs or variants thereof which are particularly susceptible to denaturation and particle formation, the utility of the invention extends generally to all protein and polypeptide pharmaceuticals. BPI and active fragments and analogs thereof useful with the present invention include recombinant produced proteins such as described in U.S. Patent No. 5,198,541. Co-owned, copending patent application Theofan et al., U.S. Serial No. 08/064,693 filed May 19, 1993, which is a continuation-in-part application of U.S. Serial No. 07/885,911 filed May 19, 1992, addresses BPI-Immunoglobulin fusion proteins which are variants of BPI protein comprising at the amino terminal a BPI protein or a biologically active fragment thereof, and retaining the same biological activity of BPI protein. Particularly preferred BPI materials include recombinant produced polypeptides produced according to the method of co-owned and copending Theofan et al. U.S. Application Serial No. 08/013,801 filed February 2, 1993 and entitled "Stable Bactericidal/Permeability-

Increasing Protein Products and Pharmaceutical Compositions Containing the Same," the disclosure of which is herein incorporated by reference. A preferred BPI fragment is characterized by about 1 to 199 or about 1 to 193 of the amino-terminal amino acid residues of the mature human BPI molecule as set out in Gray et al., J. Biol. Chem., 264, 9505-9509 (1989) except that residue 185 is glutamic acid rather than lysine as specified in Gray. The recombinant expression product of DNA encoding BPI amino acids 1 to 199 has been designated $rBPI_{23}$. The recombinant expression product of DNA encoding BPI amino acids 1 to 193 has been designated rBPI(1-193). A preferred BPI fragment analog comprises the first 193 amino acid residues as set out in Gray except that residue 185 is glutamic acid rather than lysine and the cysteine at position 132 is replaced with a non-cysteine residue such as alanine. Such a protein is designated $rBPI_{21}\Delta cys$ or $rBPI(1\text{-}193)ala^{132}$.

Example 1

In this example, tests of various surfactant systems were conducted to determine their utility for surface stabilization of a polypeptide pharmaceutical ($rBPI_{23}$). The $rBPI_{23}$ was provided at a concentration of 1 mg/mL in citrate buffered saline (0.02 M citrate, 0.15 M NaCl, pH 5.0). Various surfactants were then added to this preparation in order to determine their utility as stabilizers.

According to this test, $rBPI_{23}$ [BR-1] characterized by about 1 to about 199 of the first 199 amino acids of the mature human BPI molecule and produced according to the methods of Theofan et al., U.S. Patent Application Serial No. 08/013,801 filed February 2, 1993 was filled by hand to 5 mL in scaled sterile 6 mL molded glass vials (total capacity 8.4 mL, Wheaton) in the desired formulation buffer. The vials to be tested were set horizontally on a flat bed shaker (S/P rotor V) and fixed to the shaker by tape. Vials were then shaken at 150 rpm at room temperature. At 0 hours, 2-4 hours, and 18 hours, 150 μl samples were withdrawn in a biosafety cabinet using a 1 mL syringe fitted with

a 21 gauge needle. The starting, in process, and ending soluble $rBPI_{23}$ concentrations were determined by an ion exchange HPLC assay and visual observation of cloudiness of the solution was also recorded. The results are shown below in Table 1 in which acceptable stability was determined by visual inspection after the shake test.

Testing of protein preparations comprising single surfactants showed good results for use of octoxynol-9 (TRITON X-100, Rohm & Haas), laureth-4, (BRIJ 30, ICI Americas), poloxamer 403 (PLURONIC P123, BASF Wyandotte) and telomere B monoether with polyethylene glycol (ZONYL FSO-100, E.I. DuPont de Nemours). While these surfactants are capable of reducing surface tensions to low levels, they are not included in approved parenteral pharmaceuticals due to suspected toxic effects or unknown biocompatibility.

Testing of other surfactants as shown in Table 1 shows that surfactants producing a surface tension lower than 35 mN/m are capable of stabilizing rBPI at surfactant concentrations of 0.1%. This example further shows that both polysorbate 80 (TWEEN 80) and poloxamer 188 (PLURONIC F-68) were incapable of stabilizing the protein preparation alone under the shake test conditions employed. The incorporation of polysorbate 80 did, however, have the effect of clarifying a cloudy solution of BRIJ 30 which is not readily water soluble without the help of an additional solubilizer.

TABLE 1

Exp No.	Surfactant Used	Surface Tension mN/m at 0.1% Conc. at Room Temp. in Water (w), Buffer (b)[1]	Surfactant Concentration in Form. Buffer	Visual Observation		$rBPI_{23}$ Conc. by HPLC (mg/mL)			Stability as Determined by Visual Inspection
				3-4 hr	18 hr	0 hr	3-4 hr	18 hr	
1	ZONYL FSO-100	17[(w)]	0.100%	----	Clear	0.96	----	1.00	Stable
2	PS-80	41[(b)]	0.100%	----	Cloudy	1.11	----	0.02	Unstable
3	BRIJ 30	27.5[(b)]	0.500%	Cloudy	Cloudy	1.08	----	1.14	BRIJ 30 alone is cloudy.
4	TRITON X-100	32[(b)]	0.100%	Clear	Clear	1.00	1.01	0.98	Stable
5	PLUR P123	34.3[(w)]	0.100%	Clear	Clear	1.08	1.08	1.08	Stable
6	BRIJ 30/PS-80	----	0.1%/ 0.125%	Clear	Clear	1.19	1.21	1.17	Stable

TABLE 1

Exp No.	Surfactant Used	Surface Tension mN/m at 0.1% Conc. at Room Temp. in Water (w), Buffer (b)[1]	Surfactant Concentration in Form. Buffer	Visual Observation		$rBPI_{23}$ Conc. by HPLC (mg/mL)			Stability as Determined by Visual Inspection
				3-4 hr	18 hr	0 hr	3-4 hr	18 hr	
7	PLUR F-68	46[b]	0.100%	Clear	Haze	1.23	1.22	0.95	Marginal stability. Slight haze, specks.
8	PLUR F-68	44[b]	0.200%	Clear	Haze	----	----	1.04	Marginal Stability. Slight haze with a few specks.

TABLE 1

Exp No.	Surfactant Used	Surface Tension mN/m at 0.1% Conc. at Room Temp. in Water (w), Buffer (b)[1]	Surfactant Concentration in Form. Buffer	Visual Observation		$rBPI_{23}$ Conc. by HPLC (mg/mL)			Stability as Determined by Visual Inspection
				3-4 hr	18 hr	0 hr	3-4 hr	18 hr	
9	PLUR F-68/ PS-80	47[b]	0.1%/ 0.001%	Clear	Clear	1.14		1.09	Stable. Crystal clear with a few specks.

Surface tensions with superscript w are obtained from the surfactant manufacturer. Surface tensions with superscript b are obtained experimentally using Wilhelny plate method.

Example 2

In this example, additional comparisons were carried out according to the methods of Example 1 using various surfactants alone and in combination to stabilize a $rBPI_{23}$ preparation. The results are shown below in Table 2 in which acceptable stability was determined by visual inspection after the shake test. The results, particularly those of experiments 52-58 show the unexpected utility of the combination of poloxamer 188 and polysorbate 80 for stabilizing the $rBPI_{23}$ composition at concentrations where either surfactant alone is incapable of equivalently stabilizing the material under the conditions of the test. The experiments show that various combinations of concentrations of the two surfactants exhibit synergistic effects but that the preferred combination specific to $rBPI_{23}$ at 1 mg/mL concentration is that having 0.1% by weight poloxamer 188 and 0.001% by weight polysorbate 80 in citrate buffered saline (0.02 M citrate, 0.15 NaCl, pH 5.0). The results with polysorbate 80 at concentrations lower than 0.001% produced prompt cloudiness after 18 hours of shaking, but with only a small loss of protein as determined by ion-exchange HPLC MA7C column (Bio-Rad, Hercules, CA). Nevertheless, the cloudiness is unacceptable for appearance and suggests lowered stability. Testing with polysorbate 80 at concentrations of 0.005% and above all give good stability at up to 18 hours of shaking with little sign of protein loss by HPLC. Nevertheless, these higher concentrations of polysorbate 80 may provide less stability during long-term storage at 4°C and at stress temperatures of ambient room temperature or above.

TABLE 2

Exp No.	Surfactant Used	Surfactant Conc. in Form. Buffer	Visual Observation		Conc. by HPLC (mg/mL)			Stability as Determined by Visual Inspection
			3-4 hr	18 hr	0 hr	3-4 hr	18 hr	
1	ZONYL FSO-100	0.100%	----	Clear	0.96	----	1.00	Stable
2	PS-80	0.100%	----	Cloudy	1.11	----	0.02	Unstable
3	Dextran Sulfate	1 mg/mL	----	Cloudy	----	----	0.00	Unstable
4	Glycerol	10.0%	----	Cloudy	0.86	----	0.02	Unstable
5	HSA	5.0%	----	Cloudy	0.92	----	0.00	Unstable
6	Control- 5 mL Fill Volume	----	----	Cloudy	1.13	----	0.03	Unstable
7	Control 8.4 mL (complete) Fill Volume	----	----	Clear	1.13	----	1.04	Stable. One speck of precipitate.

TABLE 2

Exp No.	Surfactant Used	Surfactant Conc. in Form. Buffer	Visual Observation		Conc. by HPLC (mg/mL)			Stability as Determined by Visual Inspection
			3-4 hr	18 hr	0 hr	3-4 hr	18 hr	
8	Control- 5 mL (partial) Fill Volume	----	Cloudy	Cloudy	1.16	0.21	0.00	Unstable
9	TRITON X-100	0.500%	Clear	Clear	1.04	0.99	1.11	Stable
10	PS-80	0.500%	Clear	Cloudy	1.12	0.95	0.59	Unstable
11	PLURONIC P123	0.500%	Clear	Clear	1.15	----	1.13	Stable
12	BRIJ 30	0.500%	Cloudy	Cloudy	1.08	----	1.14	BRIJ 30 alone is cloudy.
13	TRITON X-100	0.100%	Clear	Clear	1.00	1.01	0.98	Stable

TABLE 2

Exp No.	Surfactant Used	Surfactant Conc. in Form. Buffer	Visual Observation		Conc. by HPLC (mg/mL)			Stability as Determined by Visual Inspection
			3-4 hr	18 hr	0 hr	3-4 hr	18 hr	
14	TRITON X-100	0.010%	Slt. Haze	Cloudy	0.96	0.84	0.04	Unstable
15	PLURONIC P123	0.100%	Clear	Clear	1.08	1.08	1.08	Stable
16	PLURONIC P123	0.100%	Clear	Clear	1.23	1.26	0.94	Stable
17	PLURONIC P123	0.050%	Clear	Slt. Haze	1.21	1.18	1.11	Unstable
18	PLURONIC P123	0.010%	Cloudy	Cloudy	1.14	0.00	0.00	Unstable
19	BRIJ 30/ PS-80	0.1%/ 0.125%	Clear	Clear	1.19	1.21	1.17	Stable
20	BRIJ 30/ PS-80	0.075%/ 0.094%	Clear	Clear	1.22	1.20	1.18	Stable

TABLE 2

Exp No.	Surfactant Used	Surfactant Conc. in Form. Buffer	Visual Observation		Conc. by HPLC (mg/mL)			Stability as Determined by Visual Inspection
			3-4 hr	18 hr	0 hr	3-4 hr	18 hr	
21	BRIJ 30/ PS-80	0.03%/ 0.038%	Slt. Haze	Cloudy	1.20	1.05	0.41	Unstable
22	BRIJ 30/ PS-80	0.01%/ 0.013%	Cloudy	Cloudy	1.14	0.48	0.00	Unstable
23	PLURONIC F68	0.100%	Clear	Slt. Haze	1.23	1.22	0.95	Marginal Stability
24	PLURONIC F68	0.100%	Clear	Slt. Haze	----	----	1.00	Marginal Stability
25	PLURONIC F68	0.150%	Clear	Slt. Haze	----	----	1.06	Marginal Stability
26	PLURONIC F68	0.200%	Clear	Slt. Haze	----	----	1.04	Marginal Stability

TABLE 2

Exp No.	Surfactant Used	Surfactant Conc. in Form. Buffer	Visual Observation		Conc. by HPLC (mg/mL)			Stability as Determined by Visual Inspection
			3-4 hr	18 hr	0 hr	3-4 hr	18 hr	
27	PLURONIC F68	0.300%	Clear	Slt. Haze	----	----	1.10	Marginal Stability
28	PLURONIC F68	0.500%	Clear	Slt. Haze	----	----	1.08	Marginal Stability
29	PLURONIC P123	0.070%	Clear	Clear	1.06	1.08	0.97	Marginal Stability
30	BRIJ 30/ PS-80	0.05%/ 0.063%	Clear	Clear	1.04	1.01	1.01	Stable
31	PLUR F68/ PS-80	0.1%/ 0.1%	Clear	Clear	1.05	1.06	1.10	Stable
32	PLUR F68/ BRIJ 30	0.1%/ 0.03%	Clear	Clear	1.05	1.05	1.03	Stable
33	PLUR F68/ BRIJ 30	0.1%/ 0.01%	Clear	Clear	1.06	1.04	1.05	Stable

TABLE 2

Exp No.	Surfactant Used	Surfactant Conc. in Form. Buffer	Visual Observation		Conc. by HPLC (mg/mL)			Stability as Determined by Visual Inspection
			3-4 hr	18 hr	0 hr	3-4 hr	18 hr	
34	PLURONIC F88	0.100%	Cloudy	Cloudy	1.07	0.87	0.56	Unstable
35	PLURONIC F98	0.100%	Cloudy	Cloudy	1.04	0.77	0.39	Unstable
36	PLURONIC F108	0.100%	Clear	Cloudy	1.04	0.87	0.55	Unstable
37	PLURONIC F127	0.100%	Clear	Clear	1.06	1.04	0.98	Marginal Stability
38	PLUR F68/ BRIJ 30	0.075%/ 0.01%	Clear	Clear	1.12	----	1.11	Stable
39	PLUR F68/ BRIJ 30	0.05%/ 0.01%	Clear	Clear	1.12	----	1.09	Stable

TABLE 2

Exp No.	Surfactant Used	Surfactant Conc. in Form. Buffer	Visual Observation		Conc. by HPLC (mg/mL)			Stability as Determined by Visual Inspection
			3-4 hr	18 hr	0 hr	3-4 hr	18 hr	
40	PLUR F68/ BRIJ 30	0.025%/ 0.01%	Clear	Clear	1.10	----	1.04	Stable
41	PLUR F68/ BRIJ 30	0.01%/ 0.01%	Cloudy	Cloudy	1.07	----	0.64	Unstable
42	PLURONIC F127	0.100%	Clear	Clear	1.12	----	0.93	Marginal Stability
43	PLURONIC F127	0.075%	Clear	Clear	1.10	----	0.61	Unstable
44	PLURONIC F127	0.050%	Clear	Slt. Haze	1.09	----	0.20	Unstable
45	PLURONIC F127	0.025%	Slt. Haze	Cloudy	1.07	----	0.00	Unstable
46	PLURONIC F127	0.010%	Cloudy	Cloudy	1.06	----	0.00	Unstable

TABLE 2

Exp No.	Surfactant Used	Surfactant Conc. in Form. Buffer	Visual Observation		Conc. by HPLC (mg/mL)			Stability as Determined by Visual Inspection
			3-4 hr	18 hr	0 hr	3-4 hr	18 hr	
47	PLUR F68/ BRIJ 30	0.05%/ 0.01%	Clear	Clear	1.04	----	1.01	Stable
48	PLUR F68/ BRIJ 30	0.05%/ 0.008%	Clear	Clear	1.01	----	1.01	Stable
49	PLUR F68/ BRIJ 30	0.05%/ 0.005%	Clear	Clear	1.00	----	1.03	Stable
50	PLUR F68/ BRIJ 30	0.03%/ 0.008%	Clear	Clear	1.06	----	0.99	Marginal Stability
51	PLUR F68/ BRIJ 30	0.03%/ 0.005%	Clear	Cloudy	1.01	----	0.79	Unstable
52	PLUR F68/ PS-80	0.1%/ 0.05%	Clear	Clear	1.14	----	1.11	Stable. A few specks.

TABLE 2

Exp No.	Surfactant Used	Surfactant Conc. in Form. Buffer	Visual Observation		Conc. by HPLC (mg/mL)			Stability as Determined by Visual Inspection
			3-4 hr	18 hr	0 hr	3-4 hr	18 hr	
53	PLUR F68/ PS-80	0.1%/ 0.01%	Clear	Clear	1.14	----	1.11	Stable. A few specks.
54	PLUR F68/ PS-80	0.1%/ 0.005%	Clear	Clear	1.15	----	1.10	Stable. A few specks.
55	PLUR F68/ PS-80	0.1%/ 0.001%	Clear	Clear	1.14	----	1.09	Stable. A few specks.
56	PLUR F68/ PS-80	0.1%/ 0.0005%	Clear	Cloudy	1.12	1.09	1.02	Unstable
57	PLUR F68/ PS-80	0.1%/ 0.0001%	Slt. Haze	Cloudy	1.09	1.09	1.02	Unstable
58	PLUR F68/ PS-80	0.05%/ 0.001%	Clear	Cloudy	1.08	1.00	0.72	Unstable

Example 3

In this example, a study was conducted to compare the efficacy of $rBPI_{23}$ formulated with and without the preferred formulation of the invention in an actinomycin-D sensitized mouse model according to Pieroni et al., Proc. Soc. Exp. Biol. & Med.; 133, 790 (1970). According to this example, ICR mice were administered an intravenous injection of actinomycin-D (800 μg/kg). Immediately thereafter, groups of 15 mice each received an injection of one of several doses of $rBPI_{23}$ [BR-1] characterized by about 1 to about 199 of the first 199 amino acids of the mature human BPI molecule and produced according to the methods of Theofan et al., U.S. Patent Application Serial No. 08/013,801 filed February 2, 1993 at 1 mg/mL in citrate buffered saline (0.2 M citrate, 0.15 M NaCl, pH 5.0). The mouse injections were at dosages of 0.03, 0.1, 1.0 and 3.0 mg/kg. As a control, some animals received the formulation buffer with or without the poloxamer and polysorbate surfactants. Deaths were recorded over seven days.

The results are shown in Figures 1 and 2. Figure 1 shows the number of mice surviving on each study day in the buffer and 3.0 mg/kg $rBPI_{23}$ treatment groups. For both buffer groups (with or without poloxamer and polysorbate surfactants), mortality was 80% overall. In contrast, $rBPI_{23}$ in the presence of excipients was even more potent than either buffer or $rBPI_{23}$ without excipients. Figure 2 summarizes the data for the different dose groups at day 7 (final survivors). Beginning at the 0.1 mg/kg dose level, $rBPI_{23}$ formulated with the preferred surfactant formulations provided significantly greater protection to the lethal effects of LPS ($P < 0.05$ or better) than did $rBPI_{23}$ in the absence of added excipients.

Example 4

In this example, experiments were conducted to determine the turbidity of various rBPI-containing pharmaceutical compositions with and without the preferred surfactant formulation of the invention. In this context, turbidity refers to the tendency of pharmaceutical compositions to engage in unfolding (i.e., loss of tertiary protein structure) and/or particle formation (interactions between individual proteins to form larger (> 10 μm) particles). The pharmaceutical compositions tested contained either rBPI(1-199)ala^{132}, rBPI(1-193)ala^{132} or various samples of $rBPI_{23}$ produced according to co-owned and co-pending U.S. Patent Application Serial No. 08/013,801 filed February 2, 1993 in either a citrate buffer (20 mM sodium citrate/150 mM sodium chloride, pH 5.0) or a citrate buffer containing 0.1% poloxamer 188 and 0.002% polysorbate 80.

Samples were analyzed to determine their resistance to turbidity over time at increasing temperature and at pH 7.0. Prior to analysis, all samples were diluted to a concentration of 0.1 mg/mL in 50 mM potassium phosphate at pH 7.0. Turbidity measurements were obtained by placing samples in quartz cuvettes for use in a Shimadzu UV-160 UV-Vis spectrophotometer equipped with a temperature-controlled cuvette holder attached to a recirculating water bath. Upon equilibrating the cuvette holder at 57°C, absorbance at 280 nm was measured to confirm that samples had been diluted to the proper concentration. Following this, the absorbance of samples at 350 nm was measured every 2 minutes for 1 hour to determine the change in absorbance over time.

Results are presented in Figure 3 showing a lower rate of change in turbidity (i.e., a lower rate of increase in absorbance over time), indicating increased stability against the formation of particles. As shown in Figure 3, the addition of the preferred combination of surfactants resulted in increased stability (resistance to particle formation) of all compositions tested. Moreover, the rBPI(1-199)ala^{132} and rBPI(1-193)ala^{132} exhibited greatly improved resistance to particle formation relative to wild-type compositions [$rBPI_{23}$].

Example 5

In this example surface tension measurements were made of polysorbate and poloxamer surfactants or combinations of the two in solutions of the BPI protein product $rBPI_{21}\Delta cys$ according to the procedure set out in the Krüss Digital Tensiometer K10ST Users Manual, Chapter 4: Measuring with the Plate. A decrease in surface tension indicates an increase in the surface activity of the surfactant, which has conventionally been thought to be the mechanism by which surfactants stabilize proteins. These procedures established that poloxamer surfactants provide advantageous results by a different and unexpected mechanism.

Specifically, a 2 mg/mL solution of unformulated $rBPI_{21}\Delta cys$ (lot 30216) was diluted with 20 mM sodium citrate, 150 mM sodium chloride, pH 5.0 rendering a 1 mg/mL solution. 15 mL of this solution was placed into a 50 mL glass beaker containing a mini stir bar. Surfactants poloxamer 188, polysorbate 80, or combinations of both were added incrementally up to 0.10%. Before each surface tension measurement, the platinum plate was heated above the reducing zone (blue flame) of a gas burner until the plate just began to glow red. The platinum plate was heated for about 10 to 15 seconds while turning the plate from side to side and then suspended back into the instrument. Each addition of surfactant was gently mixed using a magnetic stirrer and the solution was allowed to stand for 2 minutes on the thermostat vessel equilibrated at 4.6°C. The value for the surface tension was read after five minutes.

The first part of this experiment evaluated the surface activity of the surfactants alone in buffer. Using the citrate saline buffer (20 mM sodium citrate, 150 mM sodium chloride, pH 5.0) as the baseline, surfactants were added incrementally. Figure 4 is a plot of surface tension dependence on surfactant concentrations; the corresponding data is presented in Table 3. The open squares represent the citrate saline buffer in varying concentrations of poloxamer 188 while the closed circles represent the same buffer in varying concentrations of

polysorbate 80. The citrate-saline buffer solution alone had a surface tension of about 75 mN/m at 4.6°C, similar to H_2O. With increasing concentrations of surfactants, the buffer solution showed decreasing surface tension. With 0.10% poloxamer 188, the surface tension of the solution was 55 mN/m. On the other hand, with 0.10% polysorbate 80, the surface tension of the solution was 45 mN/m. The decrease in surface tension indicates an increase in the surface activity of the surfactant, i.e., the lower the surface tension, the higher the surface activity. The results indicate that polysorbate 80 is more surface active than poloxamer 188.

In the second part of the experiment, the surface activity of $rBPI_{21}$ Δcys in the presence of surfactants was evaluated. The results show that $rBPI_{21}$ Δcys at 1 mg/mL in citrate saline buffer, pH 5.0, is surface active with a surface tension of about 54 mN/m at 4.6°C. The addition of polysorbate 80 (PS80) alone up to 0.0005% did not change the surface tension of $rBPI_{21}$ Δcys solution either (Figure 4, closed triangles). At concentrations of polysorbate 80 exceeding 0.0005%, the surface tension of $rBPI_{21}$Δcys follows that of buffer with PS80 alone (no BPI), in which the surface tension of the solution decreases as the concentration of polysorbate 80 is gradually increased. For buffer with PS80 alone, the surface tension of 54 mN/m was reached when the PS80 concentration was increased from 0.0005%. These results indicate that when PS80 concentration is less than 0.0005%, the surface activity of the solution is dominated by $rBPI_{21}$Δcys. On the other hand, at PS80 concentration above 0.0005%, the surface activity of the solution is modulated by polysorbate 80 the addition of poloxamer 188 (F68) alone to $rBPI_{21}$ Δcys up to 0.10% did not change the surface activity of $rBPI_{21}$Δcys solution significantly (Figure 4, open triangles).

TABLE 3

	1 % F68	2 Buffer +F68 (mN/m)	3 % PS80	4 Buffer +PS80 (mN/m)	5 % F68	6 ΔCys +F68 (mN/m)	7 %PS80	8 ΔCys +0.1%F68 +PS80 (mN/m)	9 %PS80	10 ΔCys +PS80 (mN/m)
1	0.00000	75.4	0.00000	75.1	0.00000	54.2	0.00000	53.7	0.00000	54.9
2	0.00001	74.9	0.00001	66.8	0.00001	54.7	0.00001	53.4	0.00001	55.0
3	0.00003	74.3	0.00002	60.0	0.00002	54.2	0.00002	53.3	0.00002	53.2
4	0.00005	68.2	0.00003	60.0	0.00003	54.9	0.00003	53.9	0.00003	53.3
5	0.00007	65.9	0.00005	60.0	0.00004	54.8	0.00004	53.9	0.00004	52.8
6	0.00010	64.0	0.00007	57.4	0.00005	55.0	0.00005	53.5	0.00005	52.4
7	0.00013	65.8	0.00010	56.6	0.00006	55.2	0.00006	53.5	0.00006	53.3
8	0.00015	65.4	0.00015	57.2	0.00007	55.4	0.00007	53.4	0.00007	53.6
9	0.00017	66.5	0.00020	56.7	0.00008	54.8	0.00008	53.8	0.00008	53.8
10	0.00020	65.7	0.00050	55.6	0.00009	55.0	0.00010	53.4	0.00009	53.2
11	0.00023	66.0	0.00070	55.3	0.00010	54.9	0.00020	53.5	0.00010	53.5
12	0.00027	64.4	0.00100	54.2	0.00030	55.3	0.00030	53.2	0.00020	53.2
13	0.00030	63.8	0.00300	52.7	0.00050	54.5	0.00050	52.3	0.00030	53.0
14	0.00033	64.1	0.00700	49.2	0.00070	55.5	0.00070	51.5	0.00050	52.0
15	0.00037	63.1	0.01000	48.3	0.00100	54.9	0.00100	51.0	0.00070	51.2
16	0.00040	64.2	0.03000	46.5	0.00500	54.9	0.00200	50.6	0.00100	50.5
17	0.00043	61.8	0.07000	45.3	0.01000	55.4	0.00500	50.1	0.00130	50.4

TABLE 3

	1 % F68	2 Buffer +F68 (mN/m)	3 % PS80	4 Buffer +PS80 (mN/m)	5 % F68	6 ΔCys +F68 (mN/m)	7 %PS80	8 ΔCys +0.1%F68 +PS80 (mN/m)	9 %PS80	10 ΔCys +PS80 (mN/m)
18	0.00047	62.4	0.10000	45.4	0.05000	53.6	0.01000	48.6	0.00170	49.8
19	0.00050	63.1			0.10000	53.7	0.05000	45.6	0.00200	48.8
20	0.00060	61.6					0.10000	45.0	0.00500	47.7
21	0.00070	62.5							0.01000	46.7
22	0.00080	62.0							0.05000	45.4
23	0.00100	61.7							0.10000	45.0
24	0.00300	61.2								
25	0.00500	59.3								
26	0.00700	58.9								
27	0.01000	58.4								
28	0.03000	56.6								
29	0.07000	56.1								
30	0.10000	55.1								

Example 6

Protein samples were analyzed by Differential Scanning Calorimetry (DSC) to study the unfolding (or denaturation) of the protein. The starting materials for DSC analysis were identical to those used in the surface tension measurement. A series of $rBPI_{21}\Delta cys$ solutions was prepared with varying concentrations of surfactants, poloxamer 188, polysorbate 80 or combinations of both, and diluted with buffer (20 mM sodium citrate, 150 mM sodium chloride, pH5.0) to give a final $rBPI_{21}\Delta cys$ concentration of 1 mg/mL. A series of buffer solutions was also prepared with surfactants at the same concentrations as in the $rBPI_{21}\Delta cys$ solutions to serve as blanks for DSC. Each solution was filtered and placed into a 2 mL sterile plastic vial. The samples were packed into a 4°C cold box until subjected to DSC Analysis.

The behavior of $rBPI_{21}\Delta cys$ was evaluated as the temperature of the solution was gradually increased from ambient temperature to about 90°C, at a rate of 1°C per minute. As the temperature is increased two events occur. The first event is an unfolding reaction, which is endothermic, and is illustrated by an upward peak in the scans. The second event is precipitation, which is exothermic, and is depicted by a downward peak in the scans. In the scans depicted in Figs. 5, 6 and 8-10, each scan is offset to facilitate analysis of data. In the $rBPI_{21}\Delta cys$ solution not containing surfactants (Figure 5, Scan 1) the unfolding of the protein at 65°C was followed immediately by the second event, precipitation of the protein at 66 to 67°C.

With low poloxamer 188 (PLURONIC® F68) concentrations ranging between 0.001% to 0.01%, the unfolding and precipitation events are similar to the $rBPI_{21}\Delta cys$ solution without surfactants (Figure 5, Scans 2 to 5), i.e. as $rBPI_{21}\Delta cys$ unfolds, precipitation takes place immediately. With poloxamer 188 concentrations exceeding 0.05%, the unfolding of $rBPI_{21}\Delta cys$ still occurs at 65°C, but precipitation does not occur until the temperature reaches 85°C (Figure 5, Scan 6). Figure 6 shows that at poloxamer 188 concentrations between 0.01% and 0.05%, there is a gradual transition of delayed precipitation of unfolded BPI. These results suggest that at poloxamer 188 concentrations higher than 0.01%,

unfolded $rBPI_{21}\Delta cys$ can be stabilized and the occurrence of precipitation is delayed. A plot of denaturation and precipitation temperature dependence over the surfactant (poloxamer 188) concentration is shown in Figure 7. The effects of poloxamer 188 appear to delay the precipitation of $rBPI_{21}\Delta cys$ to a higher temperature but not to stabilize its native structure as the T_m (denaturation temperature) and ΔH (energy of denaturation) did not change.

$rBPI_{21}$ Δcys formulated with polysorbate 80 at concentrations up to 1% was likewise analyzed. The isotherms were similar to $rBPI_{21}\Delta cys$ solution without surfactants (Figure 8: Scans 1 and 8-13, Figure 9: Scans 11, 12). Polysorbate 80 did not maintain the $rBPI_{21}$ Δcys in solution at higher temperatures. The stabilization of unfolded $rBPI_{21}\Delta cys$ is thus unique to poloxamer 188.

The two formulations using combined poloxamer 188 and polysorbate 80, namely 0.1%F68/0.001%PS80 and 0.1%F68/0.002%PS80, showed the same scan profile as $rBPI_{21}$ Δcys containing 0.05% and 0.1% PLURONIC F68, with unfolding at 65°C and precipitation at 85°C (Figure 8: Scans 14, 15).

In addition to determining the melting behavior of $rBPI_{21}$ Δcys, rescanning was done with $rBPI_{21}\Delta cys$ formulations containing 0.05% and 0.10% poloxamer 188 to determine if unfolding is a reversible process. The temperature of the $rBPI_{21}\Delta cys$ solution was first increased to 75°C (temperature after denaturation/unfolding but before precipitation), then was cooled down for repeat scanning. Figure 10 shows that the addition of poloxamer 188 to $rBPI_{21}\Delta cys$ does not make unfolding reversible. Profiles A5,1 and A6,1 show the scanning to 75°C, while profile A5,2 and A6,2 are repeat scanning after cooling the system from 75°C. If unfolding were a reversible process, 6 and 7 scan profiles would have been obtained.

The experimental results described above demonstrate that poloxamer surfactant alone is capable of stabilizing BPI-related polypeptides in

solution and delaying the occurrence of precipitation by a mechanism that does not appear to involve modulation of the surface tension of the aqueous solution. This property is unique to poloxamer because other surfactants such as polysorbate 80 do not affect the precipitation phenomenon and do involve modulation of the surface tension of the aqueous solution.

Example 7

The rate of $rBPI_{21}$ Δcys precipitation during shipping was simulated in the laboratory by adjustment of the speed of the horizontal shaker. During five cycles of surface shipping, about 70% of the unformulated (surfactant free) $rBPI_{21}$ Δcys precipitated. By varying the speed (rpm) of the flat-bed shaker, shake tests were then constituted such that 70 to 90% of unformulated $rBPI_{21}$ Δcys subjected to the shake test precipitated. No $rBPI_{21}$ Δcys was precipitated when the unformulated product was shaken on a flat bed shaker at 110 rpm or less for 18 hours at 4°C. Shaking at 140 rpm (rather than at 150 rpm as in Examples 1 and 2) most closely simulated the agitation occurring during five cycles of surface transport. Changes in the flow dynamics of the liquid in the vial are substantially different at 140 rpm versus 150 rpm. Compositions including various concentrations of surfactant combinations were screened using the 140 rpm shake condition and the results obtained are set out in Table 4. It was determined that the optimal surfactant concentrations for protection from precipitation were 0.2% poloxamer 188 with 0.002% polysorbate 80 and 0.15% poloxamer 188 with 0.005% polysorbate 80.

Table 4
Summary of Shake Test at 140 rpm for $rBPI_{21}$ Δcys at 4°C

Poloxamer 188(%)	PS80 (%)	Visual (see note)	Concentration by MA7C HPLC (mg/ml) Before	After	Loss (%)
0.075	0.005	5	2.14	1.54	28
	0.010	4	2.10	1.56	26
	0.020	1	2.14	1.68	21
0.100	0.002	5	2.24	1.85	17
	0.005	4	2.14	1.85	14
	0.010	1	2.10	1.87	11
	0.020	1	2.13	1.94	9
0.150	0.002	3	2.19	1.92	12
	0.005	2	2.08	1.95	6
	0.010	1	2.19	1.94	11
	0.020	1	2.06	1.96	5

Table 4
Summary of Shake Test at 140 rpm for $rBPI_{21}$ Δcys at 4°C

Poloxamer 188(%)	PS80 (%)	Visual (see note)	Concentration by MA7C HPLC (mg/ml) Before	After	Loss (%)
0.200	0.002	2	2.19	1.98	10
	0.005	1	2.19	1.95	11
	0.010	1	2.22	1.95	12

Note: The scoring for visual observation is as follows:

1. Clear
2. Clear with few particulates
3. Slightly hazy
4. Hazy
5. Cloudy

Based on the above data, a preferred formulation for 2 mg/mL $rBPI_{21}$ Δcys to be stored at 4°C would contain 5 mM citrate, 150 mM NaCl, pH 5.0, 0.2% poloxamer 188 and 0.002% polysorbate 80. An alternative formulation for 2 mg/ml $rBPI_{21}$ Δcys to be stored at 4°C would contain 5 mM citrate, 150 mM NaCl, pH 5.0, 0.15% poloxamer 188 and 0.005% polysorbate 80.

In summary, aggregation/precipitation is one of the major causes of protein instability and can occur when proteins at the air-liquid interface unfold and expose hydrophobic domains. If left unprotected, proteins self-associate through the interaction of the exposed hydrophobic domains, resulting in aggregation and/or precipitation. With the use of the surfactants and surfactant combinations of the invention, protein can be stabilized in two ways. First, exposed hydrophobic regions at the air-liquid interface are shielded by poloxamer surfactants. Second, additional stabilization can be provided by polysorbate surfactants through conventional modulation of the surface activity of the solution.

Numerous modifications and variations of the above-described invention are expected to occur to those of skill in the art. Accordingly, only such limitations as appear in the appended claims should be placed thereon.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising a BPI protein or a biologically active fragment, analog or variant thereof, in combination with a polysorbate surfactant and a poloxamer surfactant.

2. The pharmaceutical composition of claim 1 wherein the poloxamer surfactant is characterized by an HLB value greater than about 14 and by having a surface tension between 10 and 70 mN/m when measured at room temperature and at a concentration of 0.1%.

3. The pharmaceutical composition of claim 1 wherein the poloxamer surfactant is poloxamer 188.

4. The pharmaceutical composition of claim 1 wherein the polysorbate surfactant is characterized by an HLB of greater than about 10 and by having a surface tension between 10 and 70 mN/m as measured at room temperature and at a concentration of 0.1%.

5. The pharmaceutical composition of claim 1 wherein the polysorbate surfactant is polysorbate 80.

6. The pharmaceutical composition of claim 1 wherein the poloxamer surfactant is present at a concentration of from about 0.01% to about 1% by weight.

7. The pharmaceutical composition of claim 1 wherein the polysorbate surfactant is present at a concentration of from about 0.0005% to about 1% by weight.

8. A pharmaceutical composition comprising a biologically active amino-terminal fragment of BPI protein or an analog or variant thereof in combination with polysorbate surfactant and poloxamer surfactant.

9. A pharmaceutical composition comprising a BPI protein or a biologically active fragment, analog or variant thereof, in combination with a poloxamer surfactant.

10. The pharmaceutical composition of claim 9 wherein the poloxamer surfactant is characterized by an HLB value greater than about 14 and by having a surface tension between 10 and 70 mN/m when measured at room temperature and at a concentration of 0.1%.

11. The pharmaceutical composition of claim 9 wherein the poloxamer surfactant is poloxamer 188.

12. A pharmaceutical composition comprising a biologically active amino-terminal fragment of BPI protein or an analog or variant thereof in combination with a poloxamer surfactant.

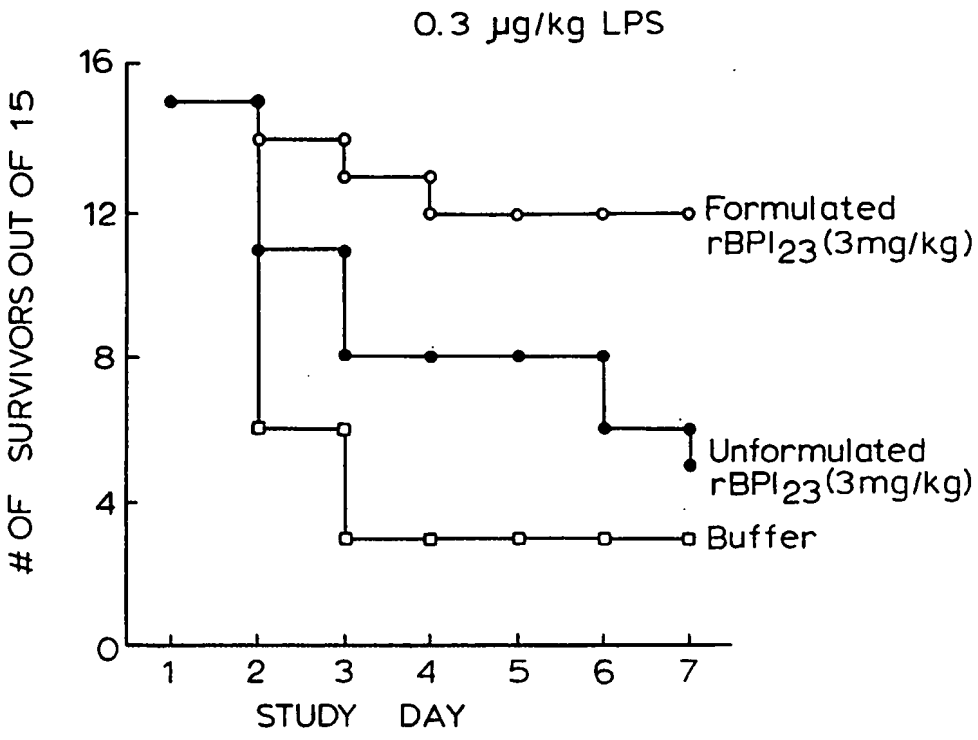
0.3 µg/kg LPS
OF SURVIVORS OUT OF 15
16
12
8
4
0
1
2
3
4
5
6
7
STUDY DAY
Formulated rBPI23 (3mg/kg)
Unformulated rBPI23 (3mg/kg)
Buffer

FIG. 1

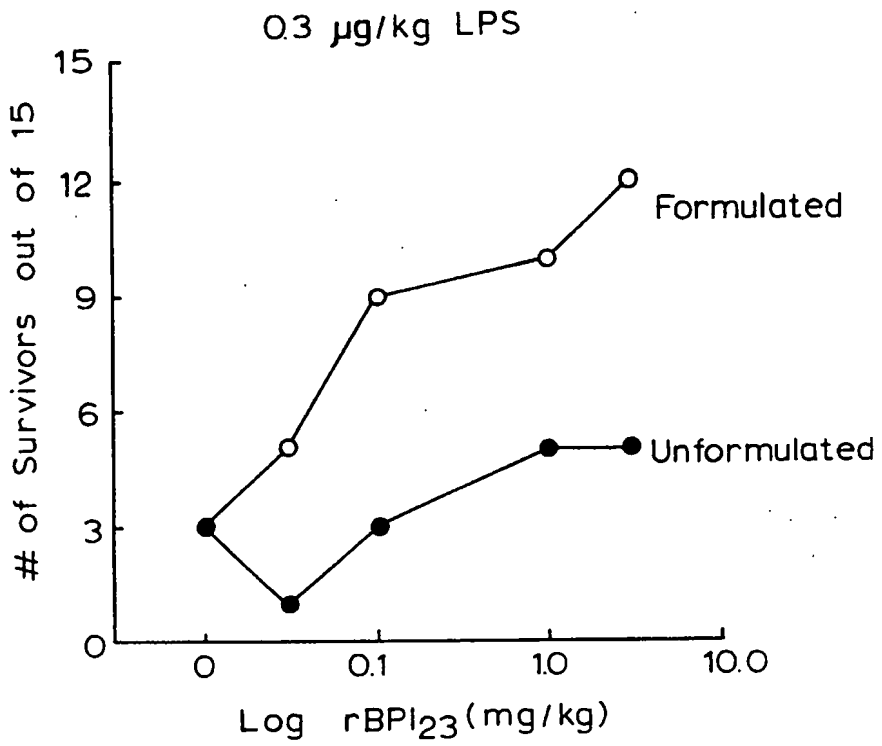
0.3 µg/kg LPS
# of Survivors out of 15
15
12
9
6
3
0
Formulated
Unformulated
0
0.1
1.0
10.0
Log rBPI23 (mg/kg)

FIG. 2

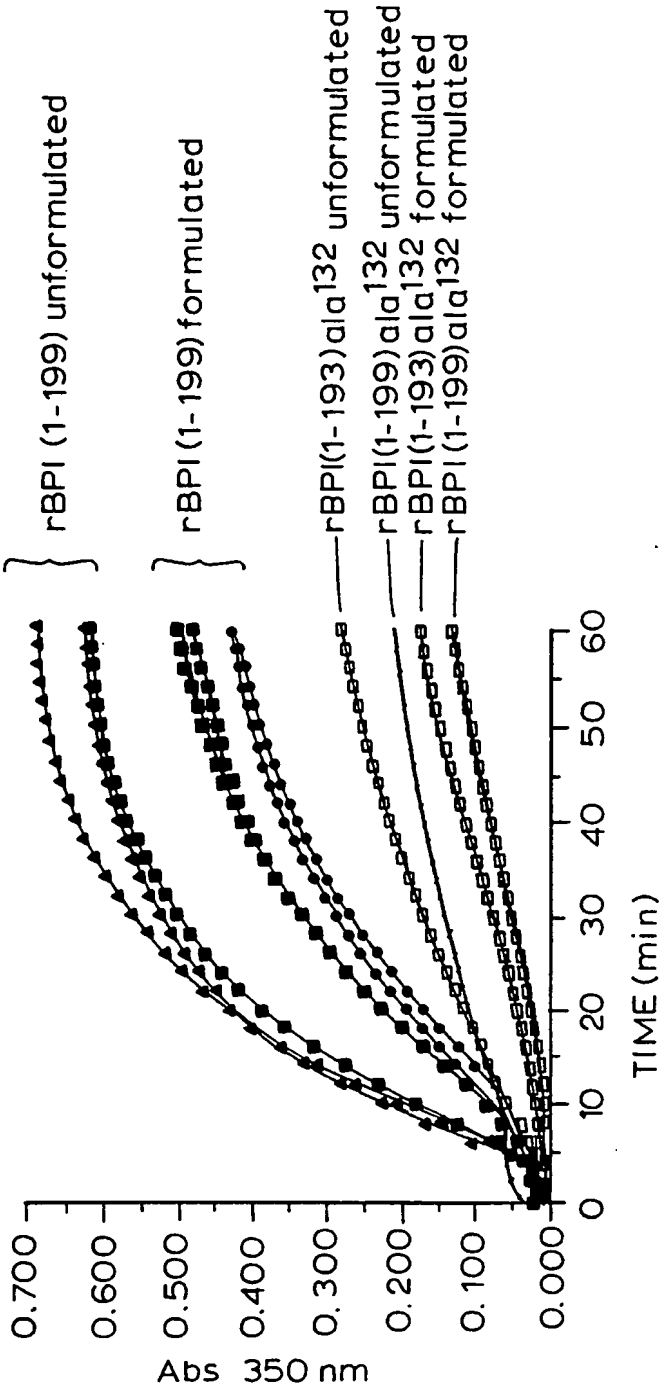
rBPI (1-199) unformulated
rBPI (1-199) formulated
rBPI(1-193)ala132 unformulated
rBPI(1-199)ala132 unformulated
rBPI(1-193)ala132 formulated
rBPI(1-199)ala132 formulated
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
Abs 350 nm
0
10
20
30
40
50
60
TIME (min)

FIG. 3

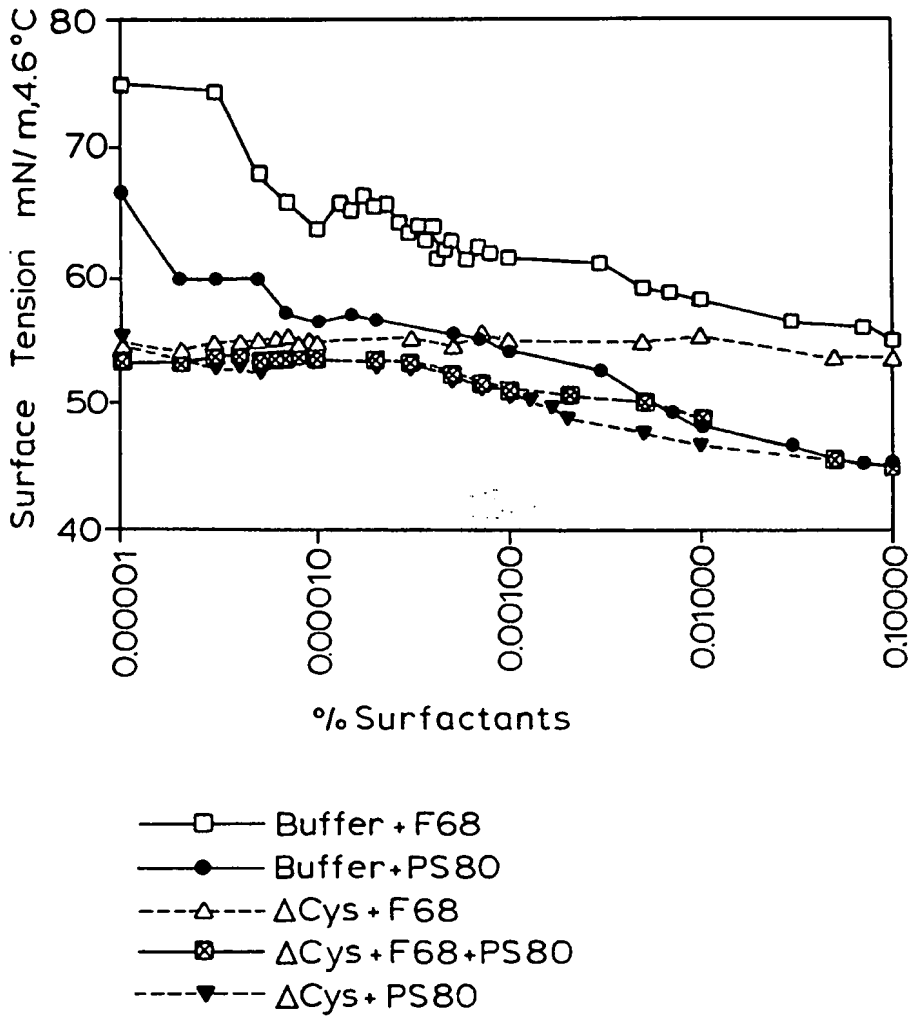
Surface Tension mN/m,4.6°C
80
70
60
50
40
0.00001
0.00010
0.00100
0.01000
0.10000
% Surfactants
Buffer + F68
Buffer + PS80
ΔCys + F68
ΔCys + F68 + PS80
ΔCys + PS80

FIG. 4

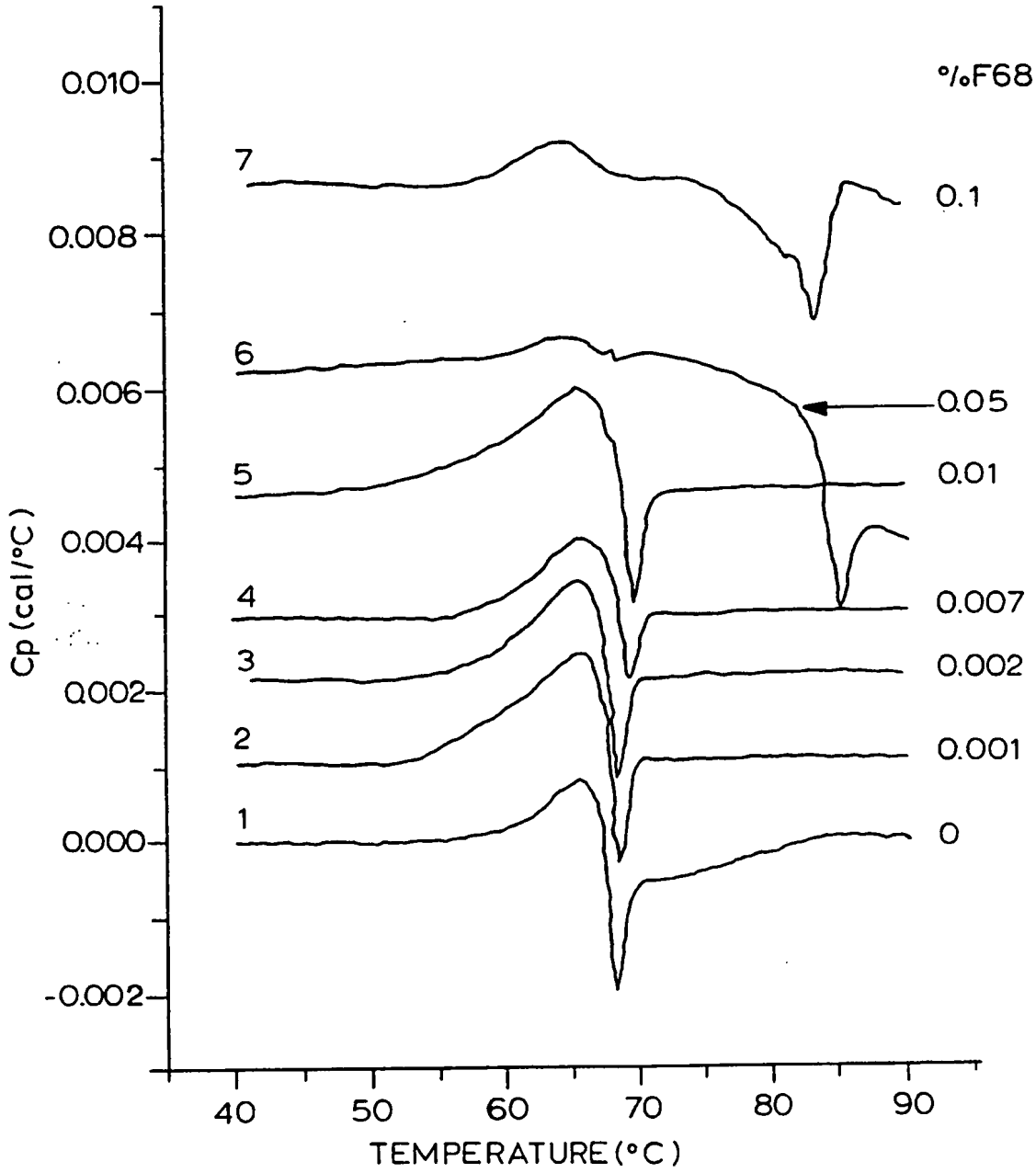
%F68
0.010
0.008
0.006
0.004
0.002
0.000
-0.002
Cp (cal/°C)
7
6
5
4
3
2
1
0.1
0.05
0.01
0.007
0.002
0.001
0
40
50
60
70
80
90
TEMPERATURE (°C)

FIG. 5

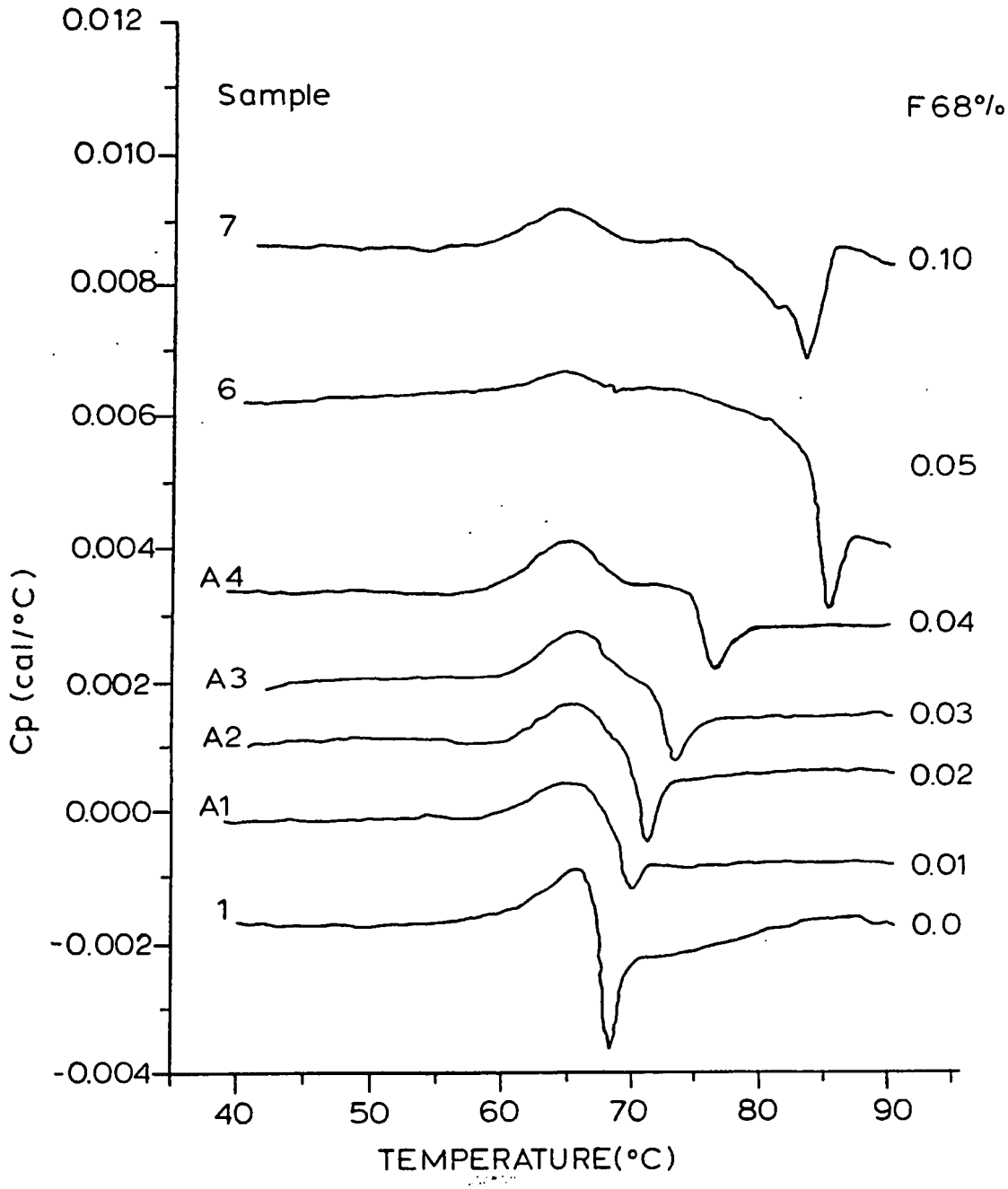
0.012
0.010
0.008
0.006
0.004
0.002
0.000
-0.002
-0.004
Cp (cal/°C)
Sample
F 68%
7
0.10
6
0.05
A4
0.04
A3
0.03
A2
0.02
A1
0.01
1
0.0
40
50
60
70
80
90
TEMPERATURE(°C)

FIG. 6

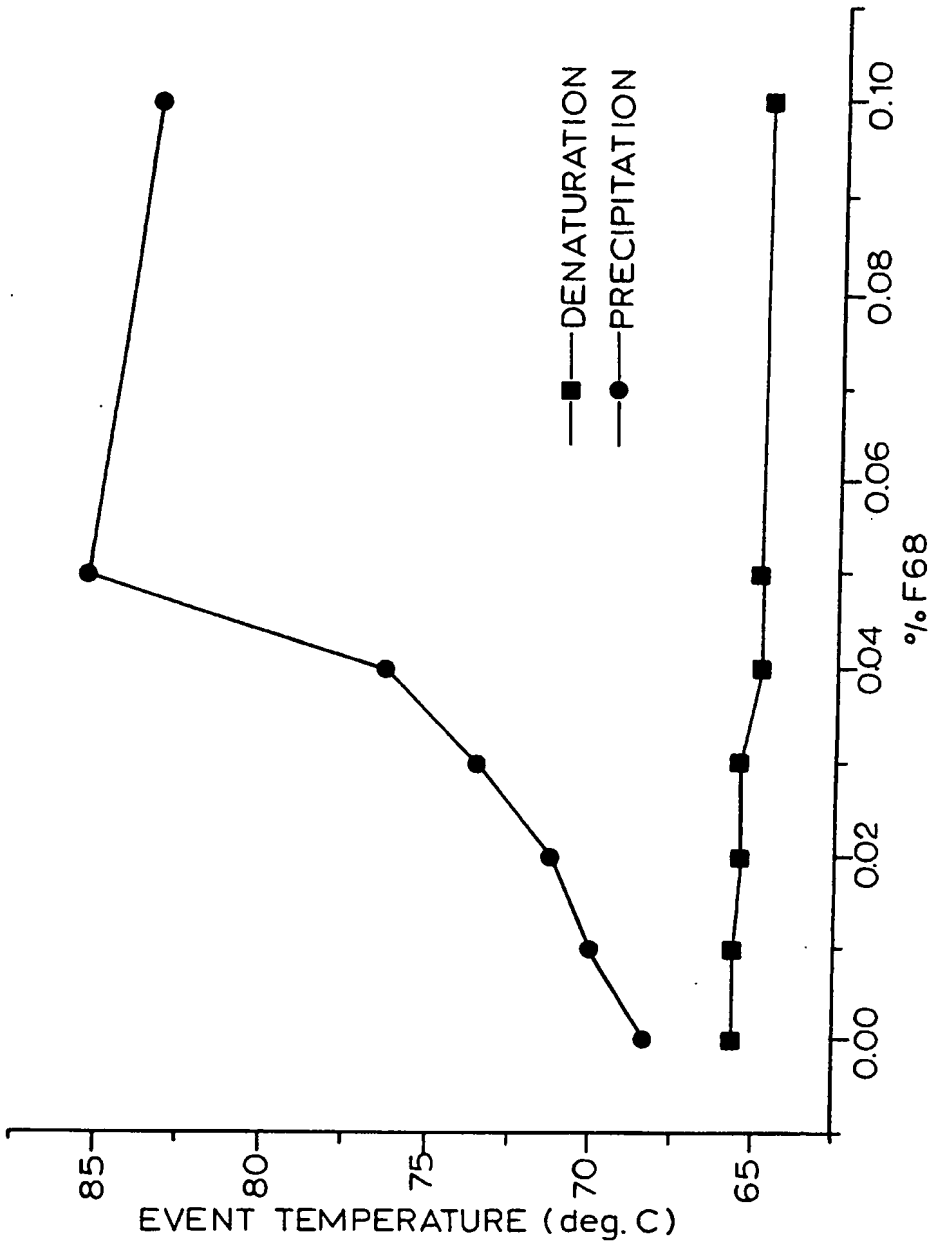
DENATURATION
PRECIPITATION
EVENT TEMPERATURE (deg. C)
85
80
75
70
65
0.00
0.02
0.04
0.06
0.08
0.10
%F68

FIG. 7

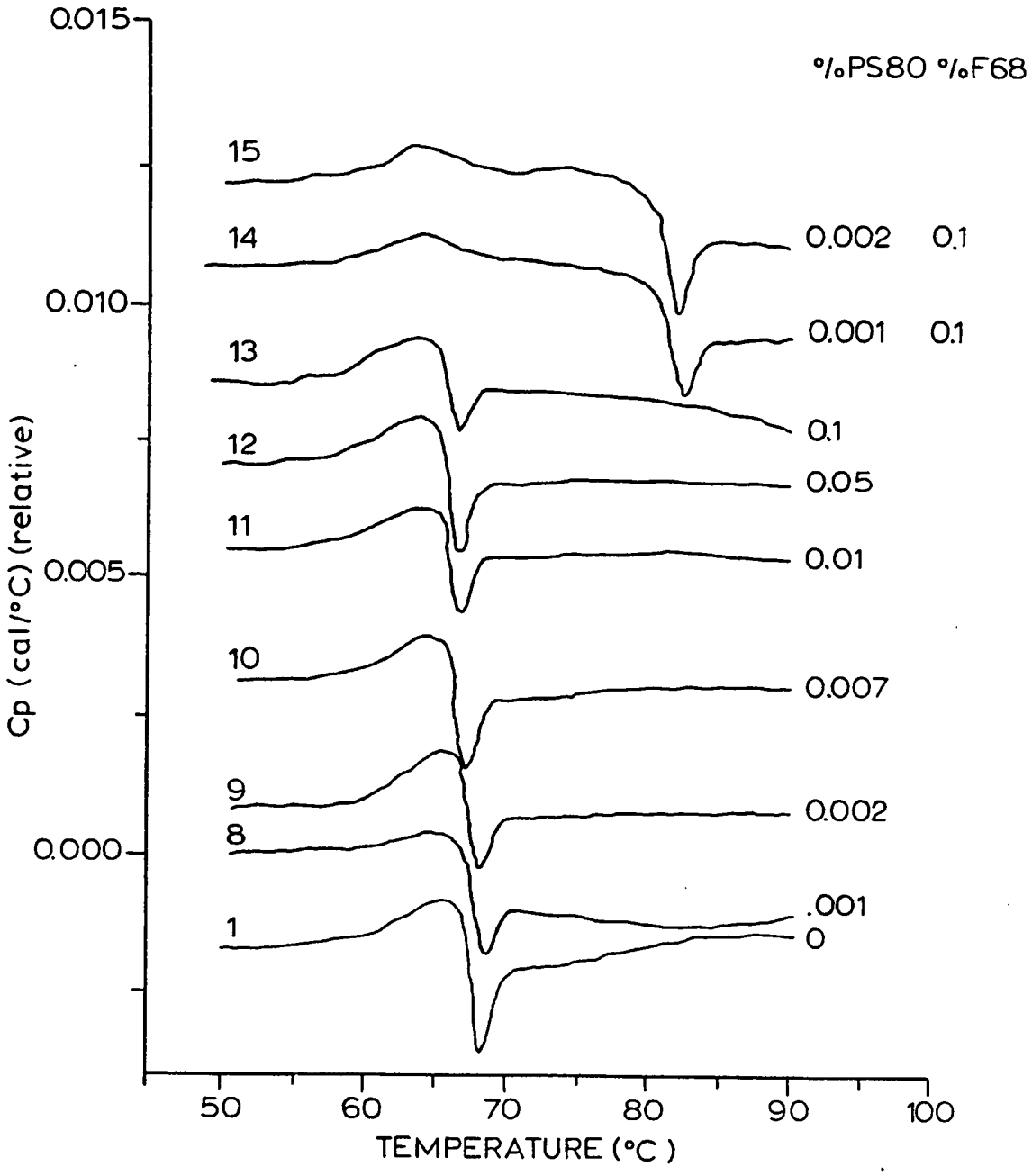
0.015
0.010
0.005
0.000
Cp (cal/°C) (relative)
%PS80 %F68
15
14
13
12
11
10
9
8
1
0.002 0.1
0.001 0.1
0.1
0.05
0.01
0.007
0.002
.001
0
50
60
70
80
90
100
TEMPERATURE (°C)

FIG. 8

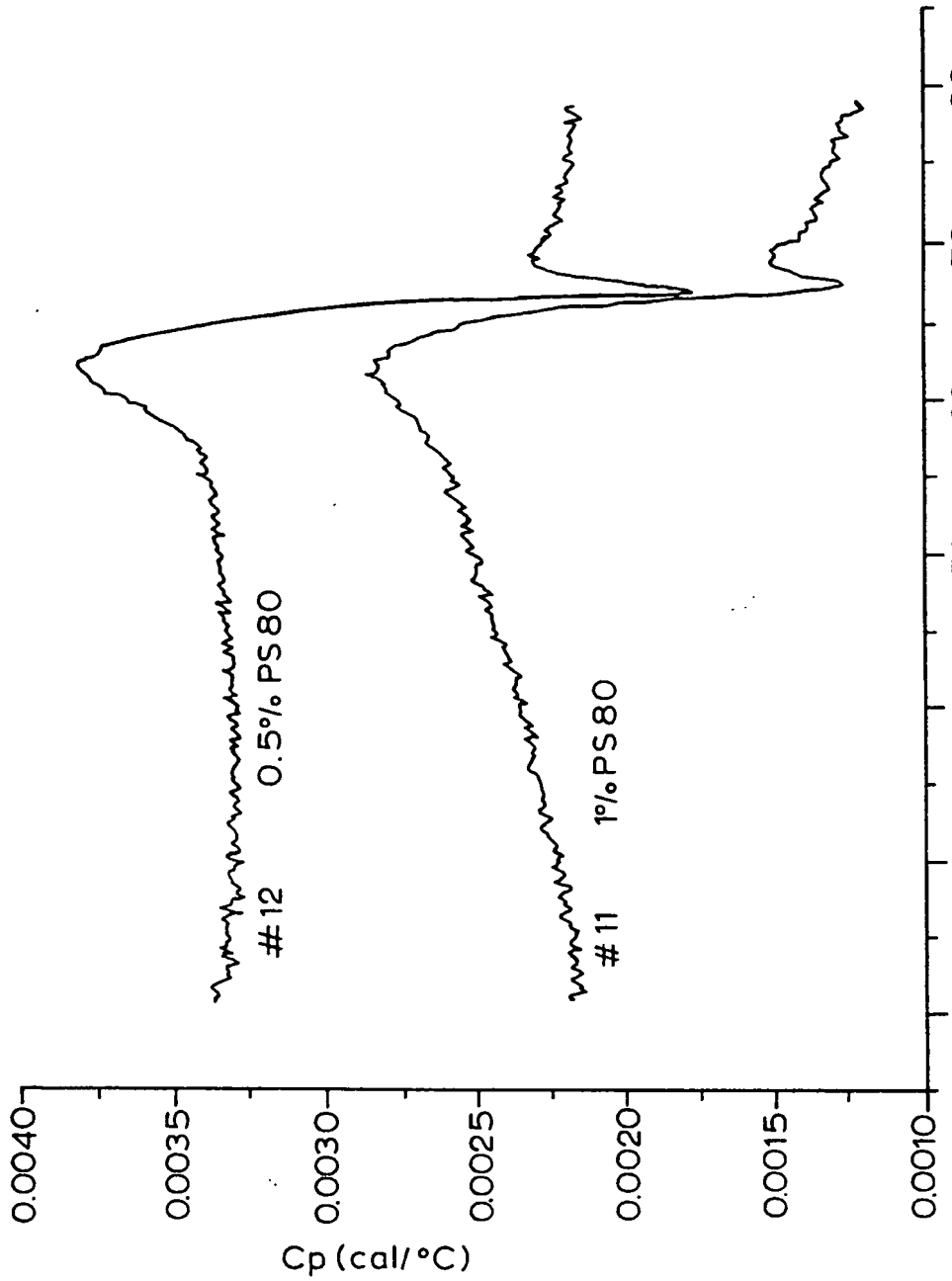
0.0040
0.0035
0.0030
0.0025
0.0020
0.0015
0.0010
Cp (cal/°C)
20
30
40
50
60
70
80
TEMPERATURE (°C)
#12 0.5% PS 80
#11 1% PS 80

FIG. 9

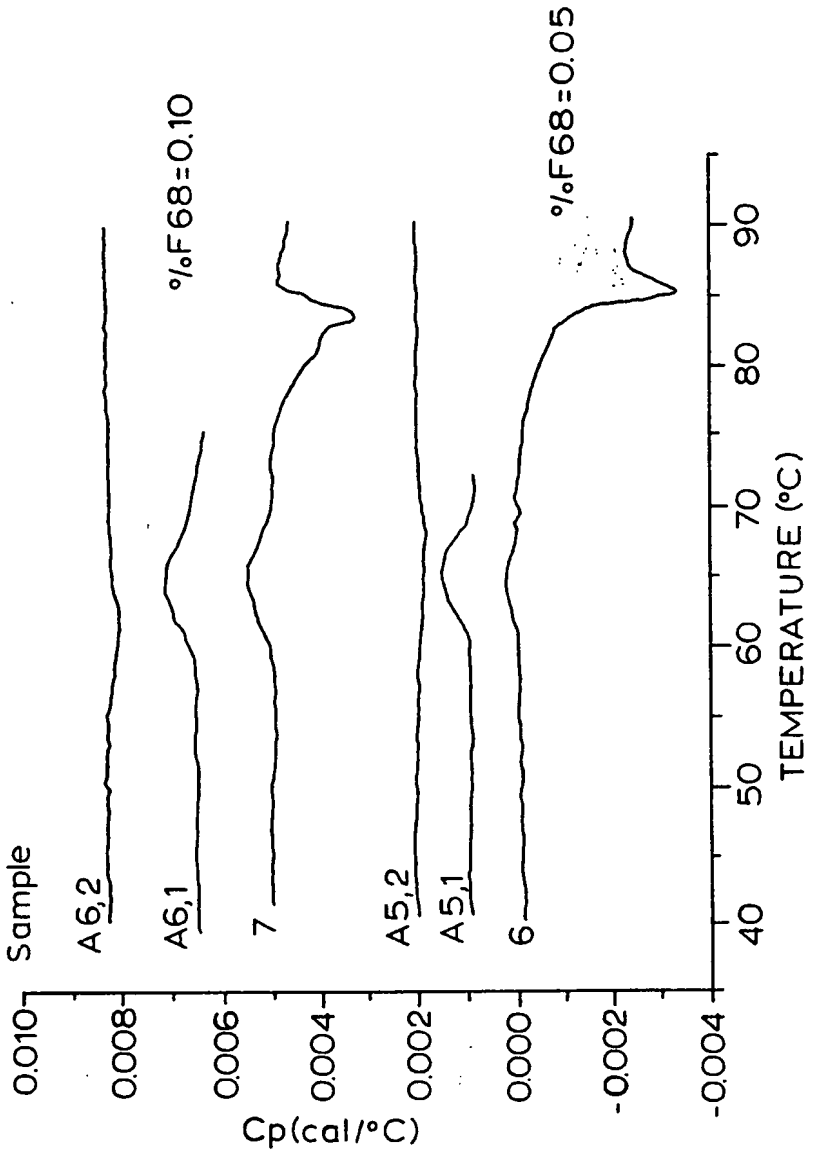
%F68=0.10
%F68=0.05
Sample
A6,2
A6,1
7
A5,2
A5,1
6
0.010
0.008
0.006
0.004
0.002
0.000
-0.002
-0.004
Cp(cal/°C)
40
50
60
70
80
90
TEMPERATURE (°C)

FIG. 10

INTERNATIONAL SEARCH REPORT

Inter. .onal Application No
PCT/US 94/01239

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K37/02 A61K47/10 A61K47/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,89 01486 (NEW YORK UNIVERSITY) 23 February 1989 cited in the application see claims 1-7 see page 10, line 22 - line 30	1-12
P,X	US,A,5 234 912 (M.N.MARRA) 10 August 1993 see claims see examples 2-4 see column 5, line 49 - line 60	1,5,7

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 May 1994	11.05.94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Scarponi, U

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter...onal Application No
PCT/US 94/01239

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8901486	23-02-89	EP-A-	0375724	04-07-90
		JP-T-	2504515	20-12-90
		US-A-	5198541	30-03-93
US-A-5234912	10-08-93	US-A-	5089274	18-02-92
		US-A-	5171739	15-12-92
		AU-A-	2699792	27-04-93
		WO-A-	9305797	01-04-93
		AU-B-	647734	31-03-94
		AU-A-	5170690	05-09-90
		EP-A-	0460058	11-12-91
		JP-T-	4506510	12-11-92
		WO-A-	9009183	23-08-90
		AU-A-	8850191	17-03-92
		EP-A-	0544832	09-06-93
		WO-A-	9203535	05-03-92